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Reagentless optical sensing of glutamine using a dual-emitting glutamine-binding protein

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Abstract

Glutamine is a major source of nitrogen and carbon in cell culture media. Thus, glutamine monitoring is important in bioprocess control. Here we report a reagentless fluorescence sensing for glutamine based on the *Escherichia coli* glutamine-binding protein (GlnBP) that is sensitive in the submicromolar ranges. The S179C variant of GlnBP was labeled at the –SH and N-terminal positions with acrylodan and ruthenium bis-(2,2′-bipyridyl)-1,10-phenanthroline-9-isothiocyanate, respectively. The acrylodan emission is quenched in the presence of glutamine while the ruthenium acts as a nonresponsive long-lived reference. The apparent binding constant, K_d', of 0.72 μM was calculated from the ratio of emission intensities of acrylodan and ruthenium (I_{515}/I_{610}). The presence of the long-lived ruthenium allowed for modulation sensing at lower frequencies (1–10 MHz) approaching an accuracy of ±0.02 μM glutamine. Dual-frequency ratiometric sensing was also demonstrated. Finally, the extraordinary sensitivity of GlnBP allows for dilution of the sample, thereby eliminating the effects of background fluorescence from the culture media.
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ABC transporters are a superfamily of proteins responsible for the active transport of various biochemical substances such as ions, amino acids, or sugars in archaea, prokaryotes, and eukaryotes [1]. In gram-negative bacteria these types of transport systems are known as binding protein-dependent permeases. These systems consist of a soluble binding protein in the periplasmic space that generally has a micromolar or submicromolar binding affinity for its substrate and two or more proteins as membrane-bound receptors [2]. The binding protein shuttles the substrate to the membrane-bound receptors that then internalize the substrate using ATP as the energy source. The soluble binding proteins in these systems have been utilized as potential sensors for various analytes including glucose [3–5], maltose [6,7], phosphate [8], and glutamine [9]. The main advantage of the binding proteins as sensors is that unlike enzymes, they do not require additional reagents [10]. The key event that accompanies molecular recognition between protein and substrate is a conformational change. Fig. 1 provides a schematic representation of this conformational change and how it is exploited in the design of an optical sensor. This figure also shows the principle of analysis for the ratiometric fluorescence-sensing technique described below.

In this paper, we report our efforts to develop a glutamine biosensor from an S179C variant of the *Escherichia coli* glutamine-binding protein (GlnBP).[1] Previous studies have shown that labeling the cysteine in position 179 with a polarity-sensitive probe such as acrylodan or anilino-naphthalene sulfonate (ANS) results in changes in the fluorescence properties of these probes in response to glutamine [9]. Although functional in this form, the methodology is far from optimal for a practical and low-cost glutamine sensor. The short lifetimes of the probes in the nanosecond range require higher frequencies (~100 MHz) and more sophisticated instrumentation to detect the glutamine-induced lifetime

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[1] *Abbreviations used:* GlnBP, glutamine-binding protein; ANS, anilino-naphthalene sulfonate; NIR, near-infrared; SDS-PAGE, sodium dedecyl sulfate–polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle medium; PBS, phosphate-buffered saline; FIA, flow injection analysis.

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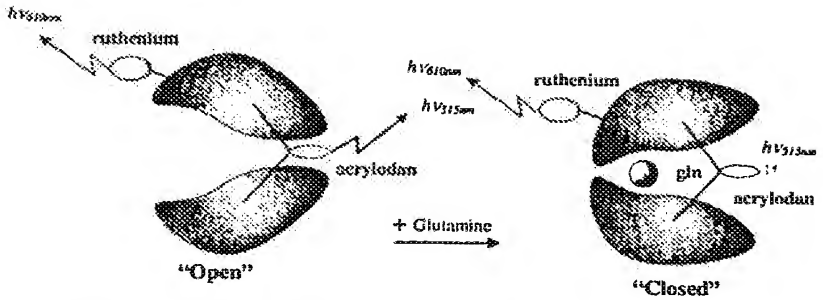

Fig. 1. Schematic representation of the principle of analysis. The acrylodan is covalently attached to a cysteine mutation on the opposite side of the glutamine-binding site. In the "open" or glutamine-free conformation the protein shields the acrylodan while in the "closed" or glutamine-bound conformation it is exposed to the solvent. This is observed as a decrease in the acrylodan intensity. In both cases, the ruthenium is unaffected by the conformational changes thereby serving as a long-lived reference.

changes. To circumvent these difficulties a method of sensing was devised where an external reference—a long-lived metal-ligand complex—was added to the solution or applied to the walls of the cuvette. The combined emission of the labeled protein and the metal-ligand complex allowed for the detection of modulation changes at lower frequencies [5,11]. We describe here an improvement on this technique by covalently linking the metal-ligand complex directly to the protein while maintaining its glutamine responsiveness (Fig. 1). The resulting dual-emitting protein can be used not only for low-frequency modulation sensing (< 10 MHz) but also for low-cost ratiometric sensing [12,13].

Glutamine sensing is very important in small and large-scale bioprocesses involving eukaryotic cell culture. Glutamine is a major nitrogen and carbon source in tissue culture media, and is considered together with glucose as a limiting factor in cell growth and product yield [14–16]. Additionally, unfavorable levels of glutamine can lead to the deleterious production of ammonia, which is toxic to cell cultures [17–19]. Monitoring of glutamine concentrations is therefore an essential aspect of process control.

Currently available glutamine biosensors rely on enzymes such as glutaminase (EC 3.5.1.2) in combination with glutamate oxidase (EC 1.4.3.11). Glutamate oxidase is required to suppress the interference of glutamic acid to the measurements [20–22]. In another assay, glutamine reacts with three different enzymes to produce NADH, which is then determined spectrophotometrically [23]. High-pressure liquid chromatograpy [24] and LC-MS-MS [25] have been used, but these techniques both require expensive instrumentation. Near-infrared (NIR) spectroscopy allows for noninvasive quantification of glutamine but requires the generation of an elaborate calibration model [26–28].

The glutamine binding protein that was used in this report has none of the disadvantages of currently available glutamine biosensors and sensing techniques. GlnBP is not an enzyme. Thus, it does not consume glutamine or require other "reagents" for its activity. The primary signal transduction mechanism involves a change in conformation (Fig. 1) from an "open" to a "closed" structure when glutamine is sequestered in the binding site [29,30]. This change in conformation is easily detected with polarity-sensitive fluorescent probes. Additionally, it is highly selective for glutamine over other amino acids and has a submicromolar binding affinity ($K_d = 0.2\,\mu M$) [9].

Materials and methods

GlnBP expression and isolation

Dr. Joseph Lakowicz generously provided the plasmid containing the S179C variant of GlnBP. Transformation and expression of the protein was carried out in *E. coli* strain HB101. To release the periplasmic GlnBP, cells from 150-ml overnight cultures were first pelleted by spinning for 10 min at 6000*g* and resuspended in 3 ml of deionized water. Three ml of chloroform was added to the cells, which were vortexed briefly and incubated for 10 min at room temperature. Then 12 ml of 20 mM phosphate buffer, pH 7.5, was added. Samples were vortexed briefly and spun for 20 min at 6000*g*. The supernatant containing the periplasmic proteins was then decanted or pipetted into a clean sterile plastic tube. The amount of S179C GlnBP was estimated to be >80% of the total periplasmic extract after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and staining with brilliant blue G (Sigma–Aldrich, St. Louis, MO). Reaction of the crude extract with 6-acryloyl-2-dimethylaminonaphthalene or acrylodan (Molecular Probes, Eugene, OR) followed by SDS–PAGE revealed close to 100% labeling of the GlnBP with almost no detectable labeling of the other proteins. This was determined by illuminating the unstained gel on a UV box to show the fluorescent band corresponding to the dye-labeled protein. A prestained standard protein ladder allowed for the estimation of the molecular weight. Thus, no

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further purification of the periplasmic extract was necessary.

Fluorophore coupling

The lone cysteine in S179C GlnBP was labeled with acrylodan as described in [9]. The labeled protein was separated from free dye by gel-permeation chromatography on a Sephadex G-25 column eluted with phosphate-buffered saline, pH 7.5. The N-terminal of the acrylodan-labeled GlnBP was selectively labeled with ruthenium bis-(2,2′-bipyridyl)-1,10-phenanthroline-9-isothiocyanate by maintaining the pH at 7.5. Ruthenium bis-(2,2′-bipyridyl)-1,10-phenanthroline-9-isothiocyanate was prepared as previously reported in [31]. The dual-labeled protein (Ru-GlnBP-Acr) was collected by elution from a Sephadex G-25 column.

Fluorescence measurements

Steady-state emission spectra were recorded on a Varian Cary Eclipse spectrofluorimeter (Varian Instruments, Walnut Creek, CA). Time-resolved luminescence decays were measured on a frequency domain fluorimeter (ISS-Koala, Champaign, IL) with the following modifications. Blue LED LNG992CFBW (Panasonic, Secaucus, NJ) driven by a current source was used as the excitation source. The modulation voltage was applied through bias T. The standard radiofrequency amplifier for the photomultiplier tubes was replaced with a ZHL-6A (Mini-circuits, Brooklyn, NY) to enhance the low-frequency performance. The excitation light was filtered by 500-, 550-, and 650 FL07 short-wave pass filters (Andover, Salem, NH). The emission light was filtered by a 500 FH90 long-wave pass filter (Andover). Luminescence decay data were analyzed by nonlinear least-squares methods.

Theory of modulation sensing

The theory of modulation sensing for combinations of short and long-lived luminophores is described in detail in [11]. For a mixture of fluorophores, the phase and modulation can be calculated using the sine and cosine transforms of the intensity decays, N_ω and D_ω, respectively, at a given frequency ω

$$N_\omega = \sum f_i m_i \sin \phi_i, \tag{1}$$

$$D_\omega = \sum f_i m_i \cos \phi_i, \tag{2}$$

where f_i is the fractional steady-state intensity, ϕ_i is the phase, and m_i is the modulation. The modulation at frequency ω is given by

$$m = \left(N^2 + D^2\right)^{1/2}. \tag{3}$$

In the case of Ru-GlnBP-Acr, the difference in lifetime between Ru and Acr is large. It is, therefore, reasonable to assume that a frequency ω can be identified where the modulation of Acr is close to 1.0 while the modulation of Ru is close to 0.0. At this frequency,

$$N = f_{\mathrm{Acr}} \sin \phi_{\mathrm{Acr}}, \tag{4}$$

$$D = f_{\mathrm{Acr}} \cos \phi_{\mathrm{Acr}}. \tag{5}$$

Using Eq. (3) we arrive at

$$m = f_{\mathrm{Acr}}. \tag{6}$$

This final conclusion proves that the modulation of a probe emitting both short and long-lived components is the fractional intensity of the short-lived component. The implication is that signal transduction need not be accompanied by lifetime changes but can be limited to intensity changes of the short-lived component. Additionally, the modulation changes are observed at frequencies lower than those required if the only emitting species is the short-lived dye as reported in [11]. These lower frequencies allow for the design of simpler, low-cost instrumentation.

Results

The absorbance spectra of Ru-GlnBP-Acr and GlnBP-Acr are shown in Fig. 2. Total protein concentration for both samples determined by the brilliant blue G-perchloric acid colorimetric assay (Sigma Diagnostics,

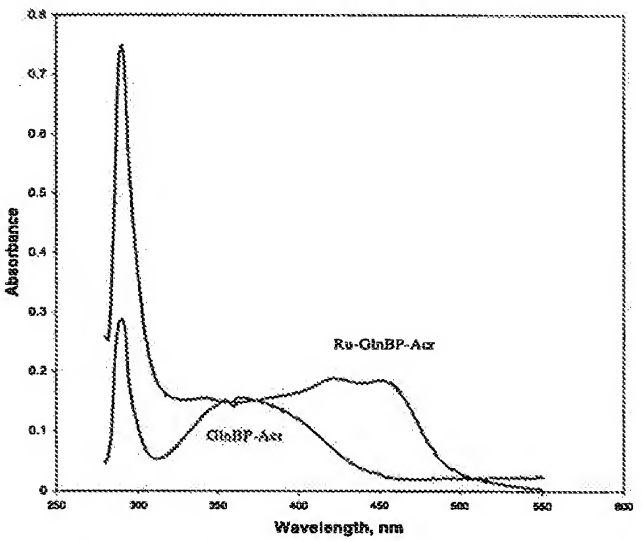

Fig. 2. Absorbance spectra of acrylodan-labeled GlnBP (GlnBP-Acr) and GlnBP labeled with ruthenium and acrylodan (Ru-GlnBP-Acr). The total protein concentrations are 9.0 μM, acrylodan is 7.8 and 7.5 μM in GlnBP-Acr and Ru-GlnBP-Acr, respectively, and ruthenium 12 μM in Ru-GlnBP-Acr.

St. Louis, MO) is 9.0 μM. The calculated amount of protein-bound acrylodan based on an extinction coefficient of 20,000 $cm^{-1}M^{-1}$ [32] is 7.8 and 7.5 μM in GlnBP-Acr and Ru-GlnBP-Acr, respectively, or about 86% labeling efficiency. This is consistent with the estimated >80% GlnBP in the periplasmic extract after SDS–PAGE as described in the experimental section. The absence of cysteine-containing proteins in the periplasm is an advantage in this case because further purification of the periplasmic fluid is not absolutely necessary. The calculated amount of protein-bound ruthenium based on an extinction coefficient of 15,000 $cm^{-1}M^{-1}$ [31] is 12.0 μM. This amount is more than the expected quantity of bound ruthenium if only the N-terminal of GLnBP was available at the reaction pH of 7.5. However, the presence of other proteins in the periplasmic extract may explain this larger value. Nonetheless, the presence of these ruthenium-labeled proteins does not interfere with the glutamine measurements because the role of ruthenium is as a nonresponsive reference. A possible drawback is that the acrylodan:ruthenium ratio may change from batch to batch and may require an initial calibration step before measurement. Further purification of the crude periplasmic extract will rectify this but may spell the difference between a low-cost and a more expensive sensor.

Fig. 3 shows the emission spectra of Ru-GlnBP-Acr in the presence of glutamine. This figure seeks to illustrate two things. The fluorescence intensity of acrylodan (λ_{max} = 515 nm) decreases with increasing concentration of glutamine as observed previously [9]. Concurrently, the luminescence intensity of Ru remains constant at λ_{max} = 610 nm. There are very few examples of small-molecule fluorescent dyes that show ratiometric emission in the presence of an analyte [33–35]. In fact, the design and synthesis of ratiometric probes are major challenges in probe chemistry. Ratiometric dyes are desirable because factors such as the concentration of the dye, the intensity of the light source, the path length, and sample positioning are internally corrected. Here we show that this is possible with a dual-emitting protein sensor. Secondly, the standard solutions of glutamine (0.1 to 6.4 mM) were prepared in glutamine-free Dulbecco's modified Eagle media (DMEM). DMEM is the most commonly used media for tissue culture. The resulting solutions were then diluted 1000× with PBS and used in the assay. The submicromolar sensitivity of the protein for glutamine remains virtually unchanged from

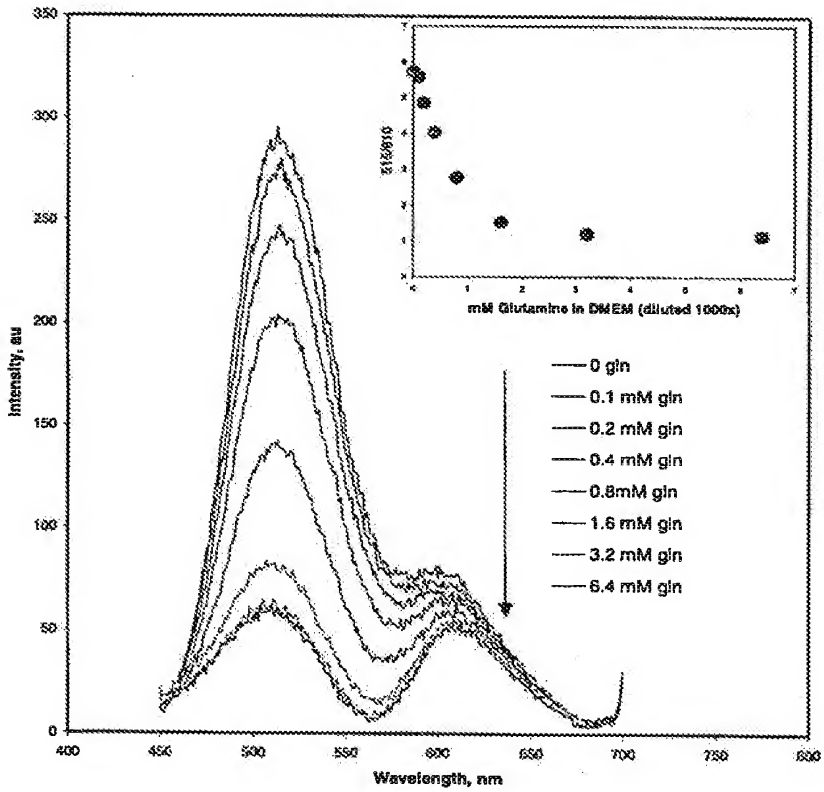

Fig. 3. Emission spectra of 2.0 μM Ru-GlnBP-Acr in increasing concentrations of glutamine. Glutamine solutions were prepared in DMEM and diluted 1000× with PBS. Excitation wavelength = 360 nm. Inset: Ratios of emission intensities at 515 and 610 nm plotted as a function of glutamine concentration.

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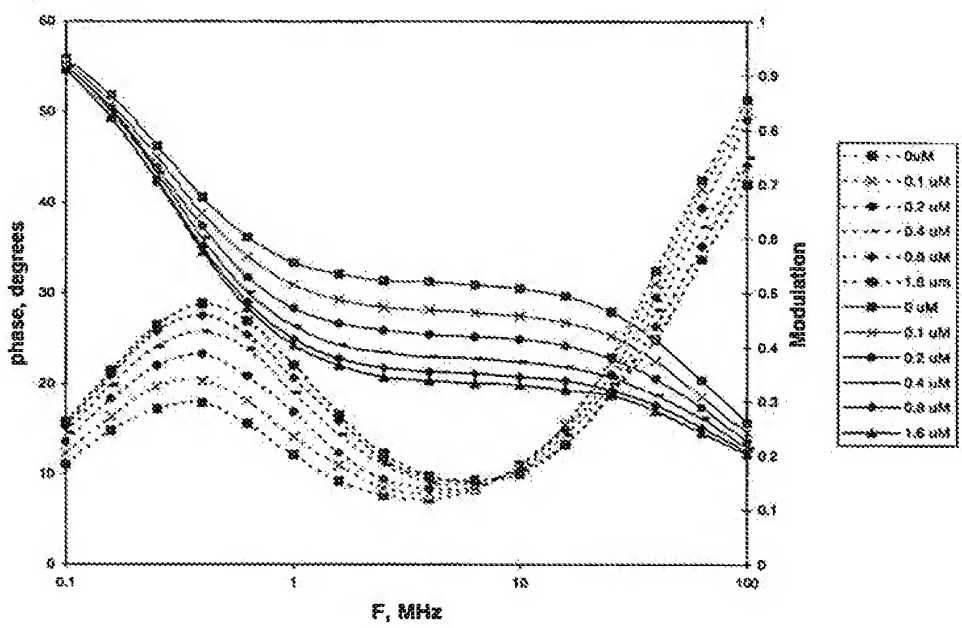

Fig. 4. Frequency-domain intensity decay traces of 5.0 μM Ru-GlnBP-Acr in 0 to 1.6 μM glutamine.

that observed with the acrylodan label alone [9]. This proves that the Ru label in the N-terminal has no effect on the binding activity of the protein. More importantly, the other components present in the complex DMEM media did not interfere with the binding of glutamine. The inset in Fig. 3 is the ratio of the intensities of acrylodan and Ru (I_{515}/I_{610}) as a function of glutamine concentration. The apparent binding constant K_d' for a single binding site was calculated from these data to be 0.72 ± 0.10 μM glutamine. This is close to the reported 100–300 nM dissociation constant for the wild type [10].

Frequency-domain intensity decay data are shown in Fig. 4. The data were fit by the least-squares method to a biexponential decay to obtain the approximate lifetimes and fractional intensities of acrylodan and ruthenium as listed in Table 1. As expected, the lifetime of acrylodan decreased from 2.4 to 1.7 ns with the binding of glutamine while the lifetime of Ru remained practically the same at about 685 ns. The ratio of the fractional intensities of acrylodan to Ru decreased from 1:1 to 1:2.

The modulation at frequencies 0.1, 2.5, 4.0, and 10 MHz is plotted as a function of glutamine concentrations in Fig. 5. Modulation measurements are easily accurate to ±0.01 [11], which leads to an accuracy of ±0.02 μM glutamine or approximately 1.4 parts per 10 billion. Although this is quite impressive, in real-life situations, modulation measurements have the disadvantage of requiring careful shielding from ambient light. This can be remedied by calculating the ratio of the modulation at two frequencies: (1) the frequency where no modulation change is detected in the presence of analyte and (2) the frequency where the biosensor emission is modulated. In the data presented here, 0.1 MHz is practically constant at all glutamine concentrations, while the data at 2.5, 4.0, and 10 MHz are responsive to glutamine levels. The inset in Fig. 5 shows the plot for the modulation ratios.

Table 1
Calculated lifetimes, τ, and fractional intensities, f, for acrylodan and ruthenium in Ru-GlnBP-Acr when the data in Fig. 3 are fit to a biexponential decay

Glutamine, μM	τ_{Ru}, ns	f_{Ru}	τ_{Acr}, ns	f_{Acr}
0.0	676	0.490	2.36	0.510
0.1	683	0.539	2.25	0.476
0.2	687	0.585	2.11	0.415
0.4	682	0.626	1.90	0.374
0.8	693	0.653	1.78	0.347
1.6	688	0.669	1.66	0.331

Discussion

Increasing demand for the production of important biological products by eukaryotic cell cultures has intensified efforts in the development of sensing devices for monitoring nutrient levels, available oxygen, and cell density in bioreactors. Glutamine together with glucose is an essential nutrient that needs to be controlled in order to maximize product formation. Here we showed an extremely sensitive glutamine sensor based on the glutamine-binding protein from *E. coli*.

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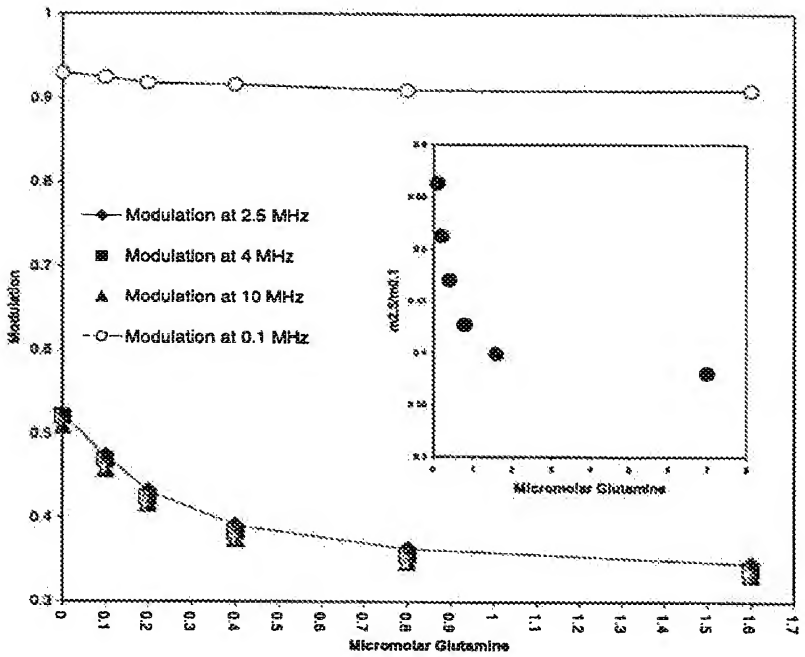

Fig. 5. Modulation data at 0.1, 2.5, 4, and 10 MHz as a function of glutamine concentrations. Inset: Ratios of modulation at 2.5 and 0.1 MHz versus glutamine concentration.

By labeling GlnBP with acrylodan at a single cysteine mutation at position 179 and ruthenium at the N-terminal, we have devised a dual-emitting biosensor that can detect submicromolar amounts of glutamine. The advantages of this new biosensor are many. From the standpoint of production, the protein can be cheaply generated because it is preferentially expressed in the periplasm of the *E. coli* host at >80% of the total protein. The only postfermentation step required is to extract the periplasmic fluid by chloroform extraction. No further purification is necessary because the lack of cysteine-containing proteins in the periplasm allows for the selective labeling of the S179C GlnBP mutant by acrylodan. Since acrylodan is responsible for the glutamine-induced signal transduction and not ruthenium, labeling of the other proteins by ruthenium in the mixture does not affect the selectivity of the sensor for glutamine.

The very high sensitivity of this protein permits the dilution of the cell culture media like DMEM by 1000× or more. At this level of dilution the interferences from other fluorescent components in the media are almost completely eliminated. Additionally, compounds that could potentially bind with the GlnBP at high concentrations are unable to compete with glutamine after dilution. With this initial dilution step, we foresee this biosensor being used in a flow injection analysis (FIA) apparatus or a microfluidic device where a tiny amount ($\leqslant 1\,\mu l$) of the sample is pumped into a mixing chamber containing either a solution of the protein or a probe with immobilized protein and a buffer solution. The mixing chamber will be designed with an optically clear window. The excitation source and detector will be positioned outside this window with no direct contact with the sample.

The dual emission of this biosensor presents several possible instrumentation designs. For example, steady-state measurements of the emission at two wavelengths with well-chosen emission filters are an improvement from simple intensity measurements at a single wavelength. The presence of the long-lived ruthenium label permits lifetime-based modulation sensing at lower frequencies. This is progress from the requisite high-frequency measurements for the short-lived acrylodan alone. Finally, modulation measurements can be further improved by dual-frequency lifetime discrimination. The latter has been demonstrated in ratiometric oxygen sensing with an instrument that utilizes low-cost semiconductor-based technology [12,13]. This method can be easily adapted to the present sensor.

As a final point, ratiometric sensing using a dual-emitting protein can be extended to other binding proteins that have been described previously [3–9] for the determination of other amino acids, sugars, and metabolites. It might also be interesting to use this technique to convert other proteins, including enzymes that undergo conformational changes in the presence of substrate, into optical biosensors.

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